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FGF22 and Its Close Relatives Are Presynaptic Organizing Molecules in the Mammalian Brain

Hisashi Umemori,[1,3] Michael W. Linhoff,[1] David M. Ornitz,[2] and Joshua R. Sanes[1,3,*]
[1]Department of Anatomy and Neurobiology
[2]Department of Molecular Biology and Pharmacology
Washington University Medical School
St. Louis, Missouri 63110
[3]Department of Molecular and Cellular Biology
Harvard University
7 Divinity Avenue
Cambridge, Massachusetts 02138

Summary

Target-derived cues promote local differentiation of axons into nerve terminals at sites of synaptic contact. Using clustering of synaptic vesicles in cultured neurons as an assay, we purified putative target-derived presynaptic organizing molecules from mouse brain and identified FGF22 as a major active species. FGF7 and FGF10, the closest relatives of FGF22, share this activity; other FGFs have distinct effects. FGF22 is expressed by cerebellar granule cells during the period when they receive synapses. Its receptor, FGFR2, is expressed by pontine and vestibular neurons when their axons (mossy fibers) are making synapses on granule cells. Neutralization of FGF7, -10, and -22 inhibits presynaptic differentiation of mossy fibers at sites of contact with granule cells in vivo. Inactivation of FGFR2 has similar effects. These results indicate that FGF22 and its relatives are presynaptic organizing molecules in the mammalian brain and suggest new functions for this family of signaling molecules.

Introduction

As the nervous system develops, axons become specialized for neurotransmitter release precisely at sites of contact with their synaptic targets, implying that target-derived factors organize presynaptic differentiation (reviewed in Sanes and Lichtman [1999]; Scheiffele [2003]; Yamagata et al. [2003]). In vertebrates, such factors have so far been identified by a candidate approach: proteins that seemed likely to promote such differentiation were tested on cultured neurons and shown to cause clustering of synaptic vesicles into aggregates resembling those found in nerve terminals. Presynaptic organizing molecules analyzed in this way include laminin β2 (Porter et al., 1995; Son et al., 1999), neuroligin (Scheiffele et al., 2000), WNT-7a (Hall et al., 2000), and TSLC1/SynCAM (Biederer et al., 2002). To date, however, no molecule has been shown to promote the initial steps in the transformation of a growth cone into a nerve terminal in vivo: WNT-7a and laminin β2 appear to be involved in maturation and stabilization of presynaptic specializations at cerebellar and neuromuscular synapses, respectively (Hall et al., 2000; Noakes et al., 1995; H. Nishimune and J.R.S., submitted), and roles of neuroligin and SynCAM in vivo remain unclear. We therefore sought molecules that play critical roles as presynaptic organizers. Rather than testing plausible candidates, however, we purified active components from mouse brain, using the ability to cluster synaptic vesicles in cultured neurons as a bioassay. We found that a major active component is fibroblast growth factor (FGF) 22.

FGFs are a family of >20 intercellular signaling molecules that signal through a set of four receptors (FGFRs; reviewed in Ornitz and Itoh [2001]). FGFs have been implicated in regulation of a wide range of processes, including cell proliferation, migration, differentiation, tissue repair, and response to injury. At least 14 FGFs and all four FGFRs are expressed in the developing or mature nervous system, and family members have been shown to play critical roles in neural induction, neural plate patterning, neuronal proliferation and survival, and neuroprotection (reviewed in Dono [2003]; Reuss and von Bohlen und Halbach [2003]). With few exceptions, however (Dai and Peng, 1995; Li et al., 2002), members of this family have not been considered as mediators of synaptic interactions.

FGF22 is part of a subfamily whose other members are FGF7 and -10; these three FGFs are more closely related to each other by sequence than to any other FGFs, and they all preferentially bind to an alternatively spliced product of the FGFR2 gene, called FGFR2b (Miki et al., 1992; Ornitz et al., 1996; Yeh et al., 2003; this study). FGF7 and -10 are expressed by mesenchymal cells and play critical roles in signaling to adjacent ectodermal cells in multiple tissues, including skin, kidney, and lung (Guo et al., 1996; Min et al., 1998). FGF22 is expressed in skin and brain but no studies on its bioactivity or function have been reported (Nakatake et al., 2001; Beyer et al., 2003).

Here we show that FGF22 promotes several aspects of presynaptic differentiation in vitro, that FGF7 and -10 share these activities, and that a member of this subfamily, most likely FGF22, is required in vivo for presynaptic differentiation of mossy fibers, the major inputs to cerebellar granule cells. Our results demonstrate a critical role for FGFs in synaptogenesis.

Results

Assay and Purification of Presynaptic Organizing Molecules

Our aim was to identify target-derived molecules that promote differentiation of neuritic segments into presynaptic nerve terminals. A striking feature of presynaptic differentiation is the aggregation of synaptic vesicles at sites of contact with target cells. We therefore used an assay based on the distribution of a synaptic vesicle-associated protein, synapsin (Murthy and DeCamilli, 2003), in cultured chick motoneurons (Henderson et al., 1996). To focus our search on molecules that exert direct effects, the motoneurons were separated from other

*Correspondence: sanesj@mcb.harvard.edu

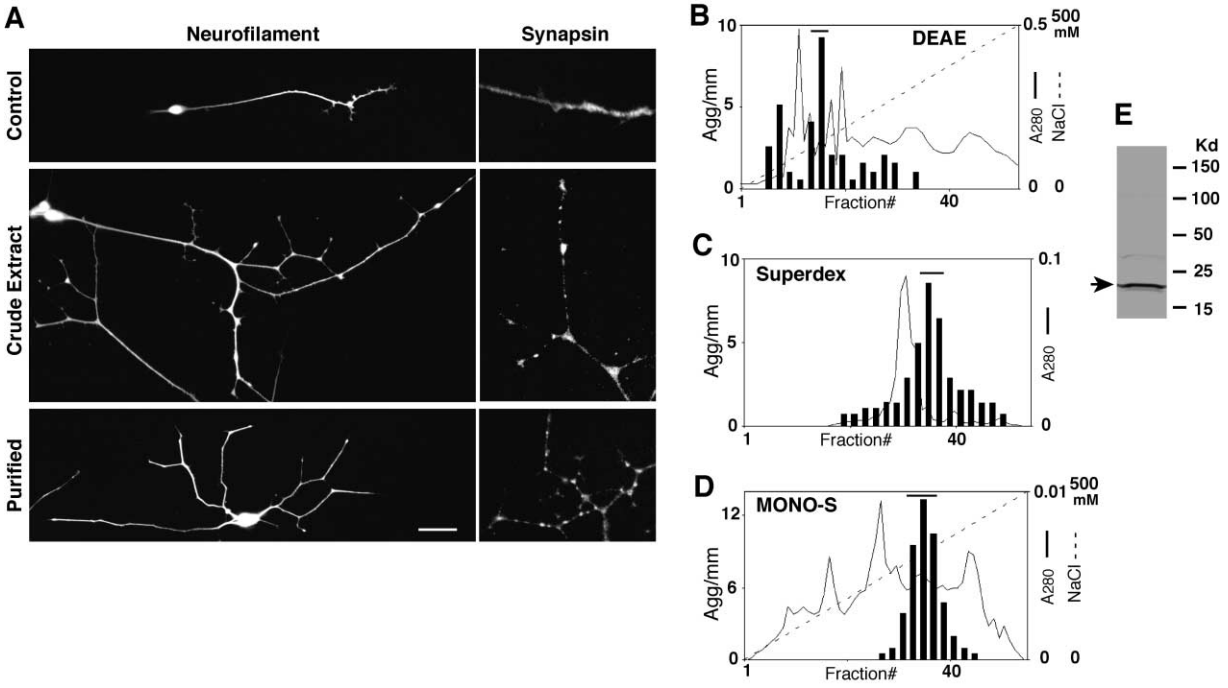

Figure 1. Purification of Presynaptic Organizing Molecules from Mouse Brain

(A) Chick motoneurons stained with anti-neurofilament to visualize neurites plus anti-synapsin to assess distribution of synaptic vesicles within the neurites. Before staining, neurons were cultured for 2 days in control medium (top) or in the presence of an extract prepared from P7 mouse forebrain (1: 200 dilution; middle) or in the presence of the purified presynaptic organizing protein shown in (E) (~100 ng/ml; bottom). Under control conditions, neurons bore a few, sparsely branched neurites and synapsin was diffusely distributed throughout the cytoplasm. The extract induced elongation and branching of neurites and aggregation of vesicles. The purified protein induced vesicle aggregation and neurite branching but not neurite elongation (see Figures 2C–2E for quantitation). Bar is 30 μm for neurofilaments, 15 μm for synapsin.

(B–D) Purification of a presynaptic organizing protein from brain extract by sequential chromatography on DEAE Sepharose FF (B), Superdex-200 (C), and MONO-S (D). Protein concentration in the eluate, monitored by absorbance at 280 nm (A_{280}) is indicated by solid lines. Note that scales in (B)–(D) extend to 0.5, 0.1, and 0.01 OD units, respectively. Salt concentration of the eluate (mM NaCl) is shown by the dotted line in (B) and (D); eluate composition was constant in (C). Presynaptic organizing activity of selected fractions, monitored by the assay in (A), is shown by vertical bars (number of aggregates per mm neurite). Fractions indicated by horizontal bars in (B) and (C) were pooled and subjected to further purification.

(E) Fractions indicated by the horizontal bar in (D) were pooled, separated by SDS-PAGE, and stained with Colloidal Blue. The major band (~20 kDa; arrow) was identified as FGF22.

neuronal cell types (≥90% purity, as shown by expression of the motoneuron marker BEN/SC1) as well as from glia (shown by complete absence of cells containing glial fibrillary acidic protein [GFAP] and S-100) and cultured at low density (~20/mm^2). Under these conditions, many neurons extended neurofilament-positive neurites within which synapsin was diffusely distributed (Figure 1A, top). We then prepared an extract from forebrains of one-week-old mice. The age was chosen because the rate of synaptogenesis is maximal during this period (Vaughn, 1989). The tissue was extracted at high ionic strength because many signaling molecules are electrostatically bound to membranes or matrix. Neurons cultured in the presence of this extract (1:200 dilution of a 2 mg/ml extract) for 2 days differed from untreated controls in three ways: their neurites were longer and more branched and synapsin was distributed in a punctate, varicose pattern (Figure 1A, middle; see also Figures 2C–2E). Thus, this extract contained molecules that affect neurite growth and may promote presynaptic differentiation.

We used synapsin distribution as an assay to monitor purification of active components from the extract. Fractionation by anion exchange chromatography (DEAE) revealed two peaks of activity that induced synapsin redistribution (Figure 1B). The second peak, on which we focus here, also increased neurite branching but did not promote neurite elongation. It was fractionated by three further chromatographic steps (Figures 1C and 1D and data not shown). The most active fractions from the final column retained both synapsin redistribution and branch-promoting activities (Figure 1A, bottom) and contained a single main protein of ~20 kDa (Figure 1E) along with minor proteins of 110 and 40 kDa (barely visible in the photograph). When neighboring fractions were assayed, the concentration of the ~20 kDa protein correlated best with synapsin redistribution activity, so it was subjected to in-gel tryptic digestion. Peptides were processed for mass spectrometry, resulting in identification of FGF22 as the major component of the sample.

Presynaptic Organizing Activity of FGF22

To verify the activity of FGF22, we cloned a mouse FGF22 cDNA, prepared recombinant FGF22, and assessed its effects on cultured motoneurons. Treatment

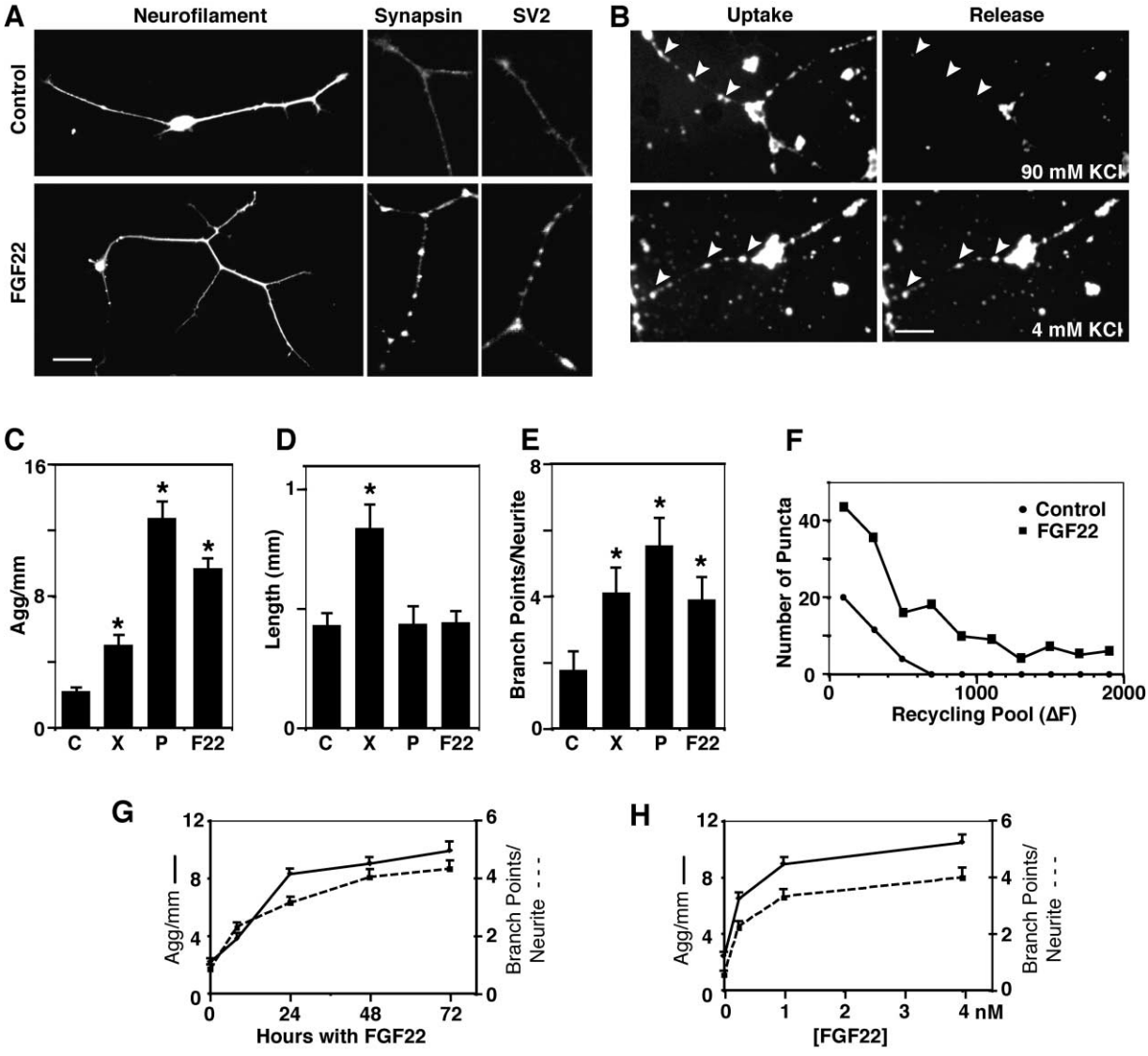

Figure 2. Presynaptic Organizing Activity of FGF22

(A) Purified recombinant FGF22 (1 nM) promoted neurite branching and clustering of synaptic vesicles in cultured neurons, as monitored by staining for neurofilament, synapsin, and SV2. Neurons were cultured with 1 μg/ml heparin. FGF22 induced synaptic vesicle aggregates and axon branching. Methods were as in Figure 1A. Bar is 40 μm for neurofilaments, 20 μm for synapsin and SV2.

(B) Vesicles in FGF22-induced varicosities are capable of depolarization-dependent recycling. Neurons were depolarized in the presence of FM1-43 dye for 90 s, then washed briefly and photographed. Varicosities (examples indicated by arrowheads) took up dye (left panels). The dye was then released by an additional round of depolarization (1 min incubation in 90 mM KCl; top right) but retained during incubation for an equal interval in 4 mM KCl (bottom right). Bar is 50 μm.

(C–E) Endogenous and recombinant FGF22 have similar effects on cultured neurons. Neurons were cultured under control conditions (C) or with crude brain extract (X), purified endogenous FGF22 (fraction shown in Figure 1E; P), or purified recombinant FGF22 (1 nM; F22). Methods were as in Figure 1A. Graphs show number of synapsin-positive vesicle clusters per mm neurite (Agg/mm; C), length of primary neurites (mm; D), and number of branch points per primary neurite (E). Endogenous and recombinant FGF22 both induced vesicle clustering and neurite branching but had no effect on neurite length. The crude extract affected all three parameters, indicating that distinct outgrowth-promoting factors were removed during the purification procedure. Bars show mean ± SEM for 500 neurons per condition. *: differs from control at $p < 0.01$ by Scheffe test.

(F) FGF22 increases the number of functional varicosities in cultured neurons. Decrease in fluorescence intensity of FM1-43-stained puncta following release, measured from micrographs such as those in (B) (top pair). All puncta in three fields were measured. FGF22 increased the total number of varicosities that take up FM1-43 on depolarization and the amount of dye each released during a second depolarization.

(G) Time course. All cultures were plated at the same time and fixed after 72 hr; FGF22 (1 nM) was added for the indicated period prior to fixation. Vesicle clustering and neurite branching were quantified as in (C)–(E). Both effects were detectable within 12 hr of FGF22 addition and near-maximal when FGF22 was present for only the last third (24/72) of the culture period.

(H) Dose dependence. Cultures were treated with FGF22 at the indicated concentration for 48 hr, then vesicle clustering and neurite branching were quantified as in (C)–(E).

of neurons with recombinant FGF22 had effects similar to those of the purified 20 kDa protein from brain: both led to formation of synapsin-rich aggregates and increased the extent of neurite branching but had no detectable effect on neurite length or neuronal survival (Figures 2A and 2C–2E and data not shown). Effects were detectable by 12 hr of FGF22 treatment and nearly maximal after 1 day (Figure 2G). After 2 days (the time used in subsequent assays), FGF22 exerted detectable effects on synapsin redistribution and branching at a concentration of 0.25 nM and near-maximal effects at 1 nM (Figure 2H). No previous studies have reported on bioactivity of FGF22, but its efficacy in our assay is similar to that reported for its close relative, FGF7, in a mitogenic assay (Post et al., 1996).

If the synapsin redistribution induced by FGF22 represents vesicle clustering, a similar effect should be seen with other vesicle components. If the vesicle clusters resemble synaptic varicosities, they should be released and recycled following depolarization. We confirmed both predictions. FGF22 redistributed the intrinsic vesicle protein, SV2, as well as the peripheral vesicle-associated protein, synapsin (Figure 2A). Moreover, neurites of FGF22-treated motoneurons took up the fluorescent styryl dye, FM 1-43 (Betz and Bewick, 1992), in a punctate pattern following depolarization with a high K^+ solution; the neurites retained the dye when subsequently repolarized but released it rapidly following a second depolarization (Figure 2B). Measurement of FM1-43 fluorescence showed that FGF22 increased both the total number of active varicosities and the amount of dye that each varicosity was capable of releasing (Figure 2F). Together, these results support the idea that FGF22 is a brain-derived presynaptic organizer.

Effects of FGF Family Members on Neurite Development

We wondered whether the effects of FGF22 on chick motoneurons were specific for this protein or shared with its relatives. To address this issue, we tested a panel of 12 purified, recombinant FGFs on motoneuron cultures, assaying the three activities described above: vesicle aggregation, axon branching, and neurite elongation. Examples are shown in Figure 3A, effects of six FGFs are quantified in Figure 3B, and all data are summarized in Figure 3C.

The two closest relatives of FGF22, FGF7 and FGF10, behaved similarly to FGF22 by all criteria assessed: all three promoted vesicle clustering and neurite branching but had no detectable effect on the length of neurites. None of the other nine FGFs tested exhibited this combination of activities. FGF4, -6, and -9 promoted vesicle clustering and neurite elongation but had no detectable effect on neurite branching. A third group, FGF17 and -18, affected neither vesicle clustering nor neurite elongation but did enhance neurite branching.

FGF1, -2, -5, and -23 had no significant effect in our assays but might exert effects under different conditions. FGF1 activates the same receptor as FGF7/10/22 (FGFR2b; see Introduction), but it also activates other receptors (Ornitz et al., 1996), which might exert counteracting effects. FGF2 has been reported to induce vesicle clustering in other systems (Dai and Peng, 1995; Li et al., 2002) and may have cell type-specific effects. FGF23 is active in mitogenic assays only at $>$100 nM concentration (D.M.O., unpublished data), so it might require still-unidentified cofactors.

We draw three conclusions from these results. First, some but not all FGFs promote vesicle clustering and might, by this criterion, be presynaptic organizing molecules. Second, FGFs differ in their activities, not only with respect to vesicle aggregation but also with respect to neurite branching and elongation. Third, there is a striking correspondence between activities of FGF family members and their primary structure, with closely related FGFs (such as FGF7, -10, and -22; FGF4 and -6; and FGF17 and -18) having similar effects (Figure 3C).

Expression of FGF22, Its Close Relatives, and Their Receptor

For FGF22 to serve as a retrograde signal for presynaptic differentiation, it must be expressed by neurons when they are receiving synapses and its receptor must be expressed by the neurons that innervate the FGF22-positive population. We used in situ hybridization to seek such pairings. We examined brains at P8, when synaptogenesis in many areas is at its peak, and at P23, by which time the pace of synapse formation has greatly declined.

FGF22 was expressed by several neuronal populations, including pyramidal neurons in hippocampus and granule cells in dentate gyrus and olfactory bulb (data not shown). Of particular interest was the cerebellum because its major neuronal populations have well-characterized synaptic inputs and targets. At P8, expression of FGF22 was high in the largest cerebellar neuronal population, the granule cells. Granule cells are born in a superficial outer granule layer perinatally, then migrate inward during the first postnatal week, whereupon they receive synapses from incoming axons called "mossy fibers" (Altman and Bayer, 1997). Both the premigratory population in the external granule layer and the postmigratory population in the internal granule layer were FGF22 positive at P8 (Figure 4A). Levels of FGF22 decreased markedly by P23, in parallel with the decline of synaptogenesis.

Although all four FGFRs are expressed in brain, we expected that the primary receptor for FGF22 would be FGFR2b based on strong evidence that its closest relatives, FGF7 and FGF10, act primarily through this alternatively spliced form of FGFR2 and bind poorly to the other main FGFR2 splice variant, FGFR2c (see Introduction). Thus, if granule cells use FGF22 to regulate differentiation of the synapses they receive, one would predict that neurons in pontine and vestibular nuclei of the brainstem, which give rise to most mossy fibers, would express FGFR2b.

As a first test of this prediction, we used a probe that recognizes all FGFR2 isoforms. As reported previously (Belluardo et al., 1997), FGFR2 is expressed by glial cells, including Bergman glia in the cerebellar cortex (Figure 4A). In addition, some neuronal populations expressed FGFR2, and these included the large pontine and vestibular neurons that give rise to mossy fibers (Figures 4B and 4C). As was the case for FGF22 in cerebellum, levels of FGFR2 RNA in pontine and vestibular nuclei declined between P8 and P23.

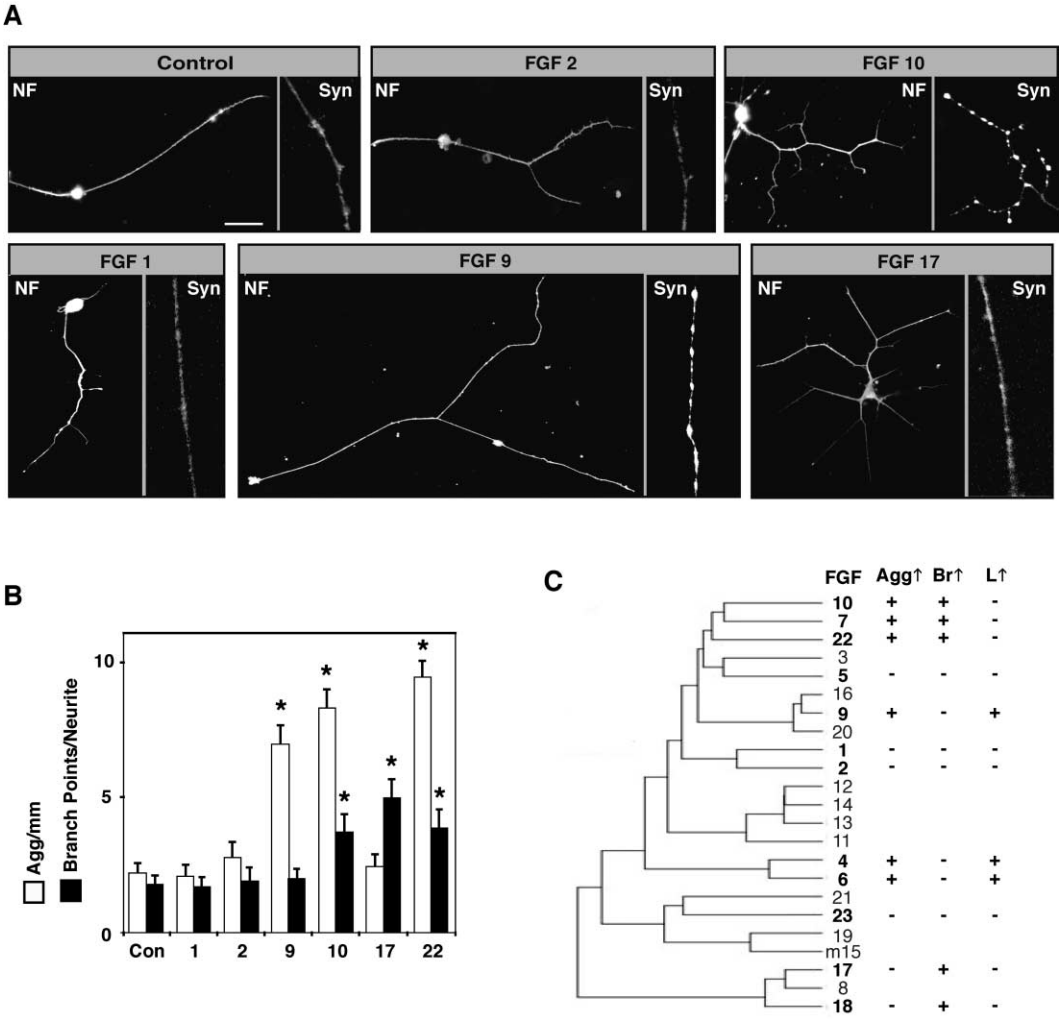

Figure 3. Diverse Effects of FGFs on Cultured Neurons

(A) Examples of neurons cultured in the presence of FGF1, -2, -9, -10, or -17. Neurons were cultured with 2 nM recombinant FGF plus 1 μg/ml heparin. After 2 days, cultures were stained with anti-neurofilament (NF) and anti-synapsin (Syn). Methods were as in Figure 1A. Bar is 40 μm for neurofilament, 20 μm for synapsin.

(B) Effects of six FGFs on vesicle clustering and neurite branching, measured as in Figures 2C–2E. Bars show mean ± SEM for 300 neurons per condition. *: differs from control at $p < 0.01$ by Tukey test.

(C) Evolutionary relationships among FGFs (from Ornitz and Itoh [2001]) and effects of 12 family members in vesicle aggregation (Agg), neurite branching (Br), and neurite elongation (L), determined from experiments such as those shown in Figures 2C–2E and 3B.

Attempts to determine whether pontine and vestibular neurons expressed FGFR2b were unsuccessful: a probe specific for the short (150 nt) sequence that distinguishes FGFR2b from FGFR2c detected this isoform in areas where total FGFR2 is abundant, but not in neurons, which express low levels of FGFR2 RNA. Moreover, an antibody specific for FGFR2b was unsuitable for immunohistochemistry. However, immunoblotting showed that FGFR2b protein was present in the pons and cerebellum (Figure 4D; multiple bands probably represent products generated by alternative splicing; Xu et al., 1998). Cerebellar FGFR2b protein levels decreased between P8 and P23, consistent with the decline in pontine and vestibular mRNA levels. We speculate that FGFR2b is generated by cells in the brainstem and transported to the cerebellum along mossy fibers.

In parallel to these studies, we examined the distribution of FGF7 and -10 because they exhibit presynaptic organizing activity in vitro and activate FGFR2b. Both were expressed at very low levels by granule cells at P8 and were undetectable at P23; FGF7 was expressed at high levels by Purkinje cells at both ages (Figure 4A). Thus, of the FGF7/10/22 subfamily, FGF22 is most likely to affect synaptic development of mossy fibers.

FGF-Dependent Presynaptic Differentiation in Cocultures

Based on activities and expression patterns described above, we hypothesized that cerebellar granule cells use FGF22 to promote presynaptic differentiation of mossy fiber axons. To test this idea, we cultured pontine or vestibular explants on laminin-coated substrates with or without dissociated granule cells. Neurites extended from the explants whether or not granule cells were

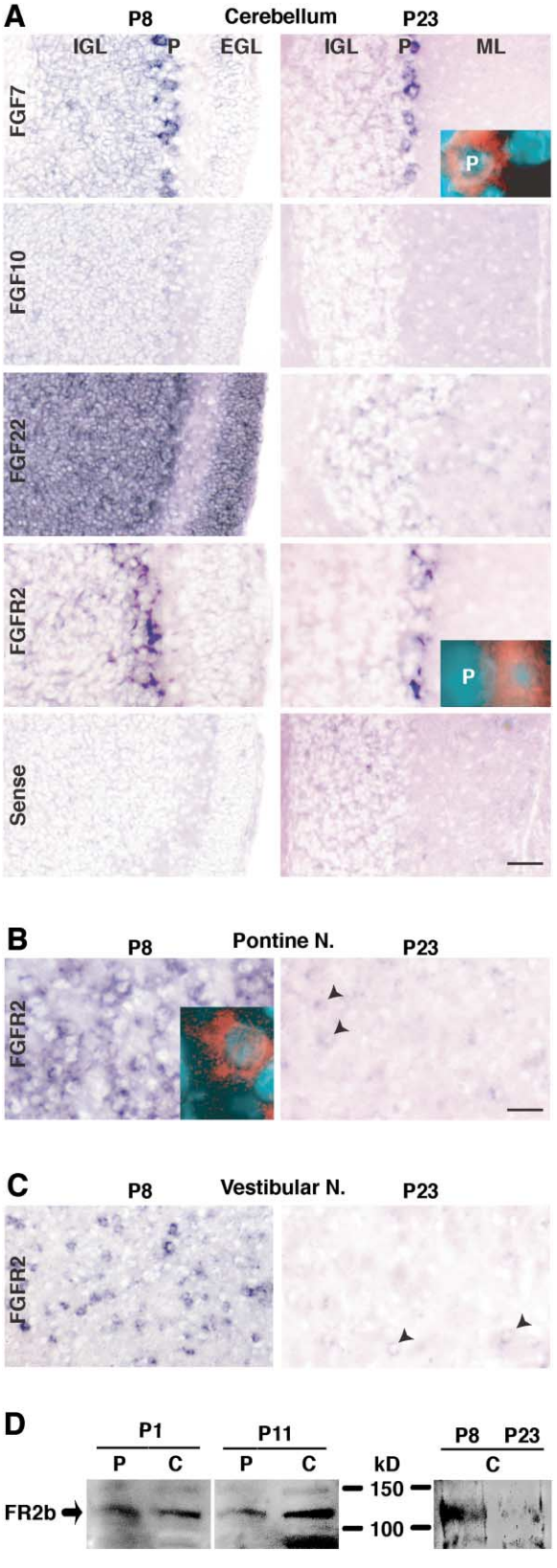

Figure 4. Expression of FGF7, -10, and -22 and FGFR2 in Brain

(A) In situ hybridization to sections of P8 (left) and P23 (right) cerebellar cortex. FGF7 is expressed in Purkinje cells at both ages. FGF10 is expressed at low levels at both ages. FGF22 is expressed at high levels by granule cells at P8; levels decline markedly by P23. FGFR2 is expressed by Bergman glial cells at both ages. Insets, at higher magnification, highlight distinction between FGF7-positive Purkinje cells and FGFR2-positive Bergman glia (red); sections were counterstained with DAPI (blue). EGL, external granule cell layer; P, Purkinje cell layer; IGL, internal granule cell layer; ML, molecular layer. Bar indicates 50 μm for low power micrographs, 10 μm for insets.

(B and C) In situ hybridization to sections of pontine (B) and vestibular (C) nuclei at P8 (left) and P23 (right). FGFR2 is expressed by large neurons in both nuclei at P8; levels decline by P23 but some cells still express low levels of FGFR2 (arrowheads). Inset: high magnification view of an FGFR2-positive pontine cell, counterstained with DAPI, shows its large size, diagnostic of neuronal identity. Bar indicates 30 μm for low power micrographs, 6 μm for high power micrographs.

(D) FGFR2b is present in postnatal pons (P) and cerebellum (C), as assayed by immunoblotting of crude lysates with an isoform-specific antibody. Levels in cerebellum decline between P8 and P23, consistent with the idea that cerebellar FGFR2b protein is associated with axons arising from vestibular and pontine neurons.

present, but the distribution of synaptic vesicles in the neurites differed in the two conditions: in the absence of granule cells, vesicle antigens were diffusely distributed, whereas in their presence, neurites extending from the explants formed vesicle-rich varicosities at sites of contact with granule cell processes (Baird et al., 1992; Scheiffele et al., 2000; Figure 5F). Using this system, we tested whether FGF22 mediated effects of granule cells.

First, we asked whether FGF22 could promote differentiation of mossy fibers in the absence of granule cells. FGF22 markedly stimulated formation of varicosities in neurites extending from pontine and vestibular neurons (Figures 5A, 5C, and 5D). FGF22 did not, however, detectably affect neurite outgrowth from either population (data not shown), nor did it significantly affect the overall levels of vesicle proteins in the neurons (Figure 5E). Thus, FGF22 appears to promote reorganization of components of the mossy fiber release apparatus into varicosities.

Next, we used the cultures to validate a method for blocking the activity of FGF22. We used a recombinant protein in which the extracellular domain of FGFR2b was rendered soluble by fusion to alkaline phosphatase (FGFR2bAP; Ornitz et al., 1992). As described above, this protein binds to, and should thereby neutralize, FGF7, -10, and -22. FGFR2bAP (100 nM) almost completely inhibited the ability of FGF22 to induce varicosities in vestibular neurites (Figures 5B and 5D). In contrast, a similar fusion protein generated from the FGFR2c isoform, which does not bind FGF7 or -10, had no effect on the ability of FGF22 to induce varicosities (FGFR2cAP; Figures 5B and 5D). These results support the idea that FGF22 binds selectively to FGFR2b and provide a means for neutralizing FGF22.

We next asked whether FGFR2bAP or FGFR2cAP affected formation of varicosities formed by vestibular or pontine neurites cocultured with granule cells. A complication in initial experiments was that antibodies to vesicle proteins stained granule cells as well as pontine or vestibular neurites. To circumvent this problem, we generated transgenic mice in which a synaptophysin-YFP fusion protein was expressed under the control of regulatory elements from the *thy-1* gene (Feng et al.,

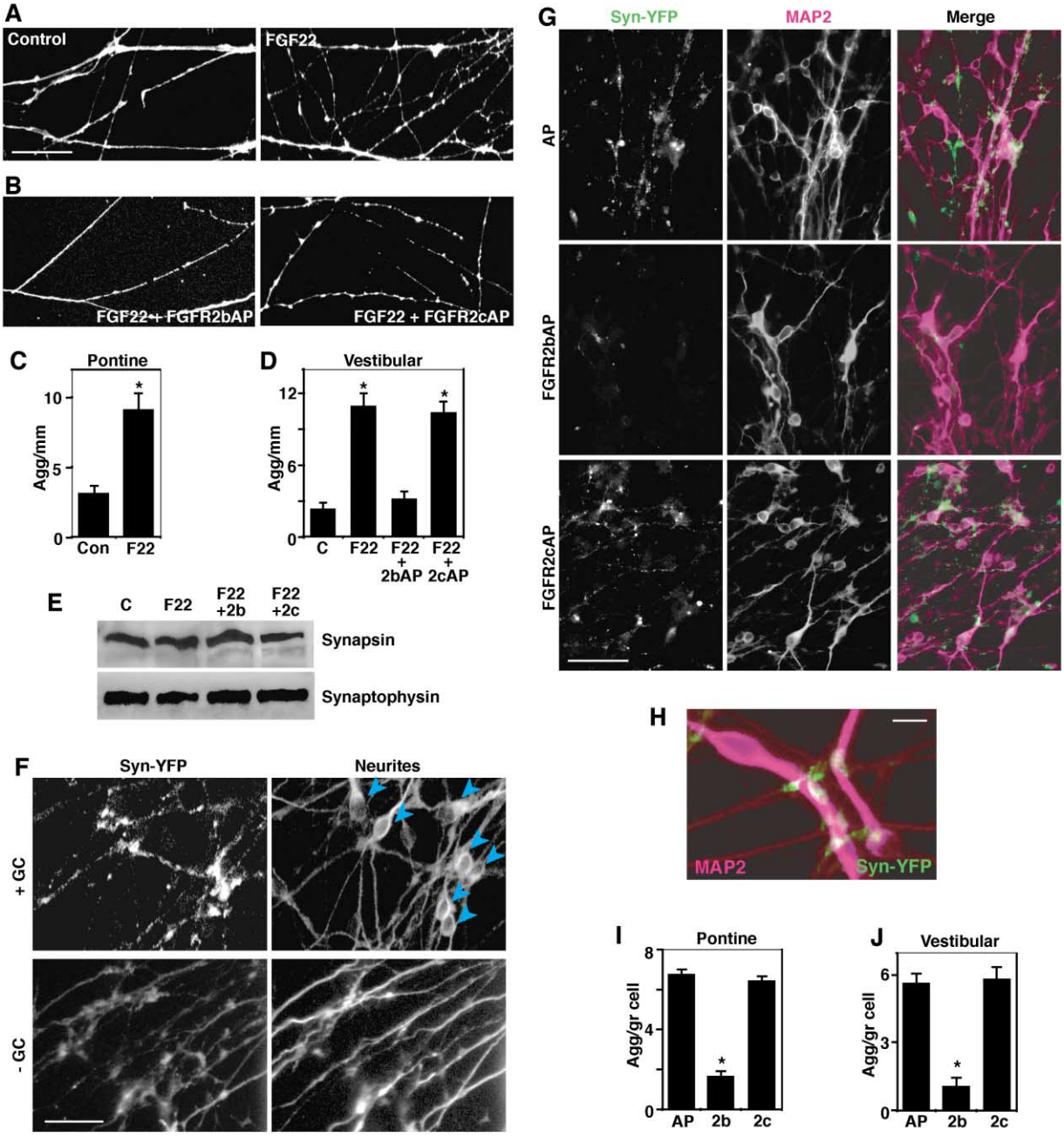

Figure 5. FGF-Dependent Presynaptic Differentiation of Pontine and Vestibular Axons In Vitro

(A and B) FGF22 (1 nM) promotes aggregation of synaptic vesicles in neurites extending from pontine explants. FGFR2bAP but not FGFR2cAP (100 nM) blocks this effect (B). Treatment and staining was as in Figure 1A. Bar is 50 μm.

(C and D) Quantitation of results from pontine explants such as those shown in panels A and B (C) and similar vestibular (D) explants. Methods as in Figures 2C–2E. Bars show mean ± SEM for 500 neurites per condition. *: differs from control at $p < 0.001$ by Student's t test (C); differs from control at $p < 0.01$ by Tukey test (D).

(E) Levels of vesicle proteins in pontine cultures, assessed by immunoblotting for synapsin and synaptophysin, are not detectably affected by treatment with FGF22, FGFR2bAP, or FGFR2cAP.

(F) Granule cells induce aggregation of vesicles in pontine neurites. (Top) Chimeric coculture of pontine explant from a mouse expressing synaptophysin-YFP (Syn-YFP) in projection neurons and dissociated granule cells (GC; marked with arrowheads) from a wild-type mouse. YFP-positive puncta form along neurites. (Bottom) Synaptophysin-YFP pontine explant grown alone; YFP is diffusely distributed in neurites. Bar is 20 μm.

(G) Chimeric cocultures were grown for 2 days in the presence of alkaline phosphatase (AP), FGFR2bAP, or FGFR2cAP, then doubly stained with antibodies to YFP and MAP2. In controls, YFP-positive puncta form predominantly at points of contact with MAP2-positive granule cells. FGFR2bAP, which binds FGF7, -10, and -22, inhibits formation of puncta. Bar is 40 μm.

(H) High-power view of neurites in a chimeric coculture, showing that synaptophysin-YFP-rich puncta are localized predominantly at sites of contact with MAP2-positive granule cell dendrites. (Pontine neurites are faintly MAP2-positive with this staining protocol.) Bar is 5μm.

(I and J) Quantitation of results from chimeric cocultures made with synaptophysin-YFP-positive pontine (I) and vestibular (J) explants. Numbers of puncta and granule cells were counted in each of ~5 20× fields per experiment. Number of granule cells per field did not differ significantly among conditions. Bars show mean ± SEM for five experiments. *: differs from control at $p < 0.01$ by Tukey test.

2000; De Paola et al., 2003) and selected lines in which the mossy fibers were YFP positive (see Figure 6A). We then generated chimeric cocultures in which pontine or vestibular explants were obtained from the synaptophysin-YFP mice and the granule cells were taken from wild-type mice. Whereas YFP was diffusely distributed when explants were cultured alone, it was redistributed into puncta in cocultures (Figure 5F), and the puncta were usually apposed to processes of granule cells (Figure 5H). FGFR2bAP inhibited formation of YFP-positive varicosities by >70% in pontine explant-granule cell cocultures and by >80% in vestibular explant-granule cell cocultures; FGFR2cAP had no significant effect on varicosity formation in either type of coculture (Figures 5G, 5I, and 5J). We also found that pontine and vestibular neurites were more extensively branched in cocultures with granule cells than in explants grown alone, and branching was decreased in the presence of FGFR2bAP. These results suggest that FGF22 or a related molecule mediates the presynaptic organizing effect of granule cells on their inputs.

To ask whether FGFR2bAP inhibited presynaptic differentiation by direct or indirect means, we performed a series of control experiments. (1) We stained cocultures with anti-neurofilament antibody, which labels pontine and vestibular neurites more intensely than granule cell neurites. FGFR2bAP had no detectable effect on number or length of pontine or vestibular neurites (Supplemental Figures S1A–S1C at http://www.cell.com/cgi/content/full/118/2/257/DC1). (2) We asked whether FGF22 or FGFR2bAP affected the morphology or vesicle distribution of granule cells cultured alone and found that it did not (Supplemental Figure S2). (3) Likewise, granule cells had similar shapes and sizes in the presence and absence of pontine explants. (4) We stained cocultures with markers for glia (anti-GFAP and S-100). Glia were present within the explants, but none were present in regions of outgrowth, where pontine neurites contacted granule cells. Neither FGF22 nor FGFR2bAP affected levels of GFAP or S-100 immunoreactivity within the explants, or the appearance of glia outside of the explants, or the level of GFAP as assessed by immunoblotting (Supplemental Figure S1D and data not shown). Together, these results support the idea that FGF22 or a closely related protein directly mediates the presynaptic organizing effects of granule cells on pontine and vestibular neurites.

FGF-Dependent Presynaptic Differentiation In Vivo

Next, we used the FGFR2bAP and FGFR2cAP fusion proteins and synaptophysin-YFP transgenic mice to ask whether an FGF22-like activity promotes presynaptic differentiation in vivo. Proteins were injected into the lateral ventricle of the brain at P3. At this time, granule cells are migrating to the internal granule layer and mossy fibers are entering this layer, but substantial synapse formation onto granule cells has not yet occurred. Animals were sacrificed at P8 when, as noted above, functional mossy fiber-granule cell synapses are numerous.

Neither FGFR2bAP nor FGFR2cAP fusion proteins had any detectable effect on the migration of granule cells (as assessed by the width of the residual external granule layer; 30 $\pm$ 3 μm in both untreated and treated cerebella), the lamination of the cerebellum, the entry of axons into the cerebellar cortex, the placement and size of Purkinje cells, or the density of granule cells (Figure 6B and data not shown). However, animals infused with FGFR2bAP had fewer synaptophysin-YFP-positive aggregates in the granule layer than uninjected animals or animals that received FGFR2cAP. As shown in Figures 6C–6E, infusion with FGFR2bAP led to significant decreases in the number, size, and intensity of synaptophysin-YFP aggregates. To ensure that the effects observed in transgenic mice reflected aggregation of endogenous presynaptic components, we repeated these experiments in wild-type mice, using antibodies to synaptophysin and synapsin to monitor presynaptic differentiation. FGFR2bAP inhibited aggregation of both endogenous proteins (Figure 6F). Western blotting revealed no significant differences between control and FGFR2cAP-treated cerebella in levels of vesicle-associated (synapsin and synaptophysin) or active zone-associated proteins (Bassoon and RIM), suggesting that the treatment affected the distribution rather than the overall amount of these proteins (data not shown). Moreover, it is unlikely that FGF signaling controls the level of synaptophysin-YFP because its expression is driven by an exogenous (*thy-1*) promoter that is highly active in numerous classes of projection neurons (Feng et al., 2000). Based on these results, we conclude that an FGF22-like activity is required for promoting presynaptic differentiation of mossy fibers in the granule cell layer of the cerebellum.

To ask whether FGFR2bAP affected presynaptic differentiation in all populations, we examined regions of the hindbrain adjacent to the cerebellum. Synaptophysin-YFP aggregates were smaller in this area than in the cerebellum, probably reflecting the fact that synaptic rosettes formed by mossy fibers are composed of multiple synaptic varicosities. FGFR2bAP had no detectable effect on vesicle aggregation in the hindbrain (Figure 6G).

FGFR2-Dependent Presynaptic Differentiation In Vivo

Effects of FGF22 on presynaptic differentiation of mossy fibers are likely to be mediated by FGFR2b. To test this idea, we asked whether effects of deleting FGFR2 were similar to those of neutralizing FGF22-like molecules. Both FGFR2 null mutants and isoform-specific FGFR2b mutants are lethal before the time that synapses form in the cerebellum (E10.5 for FGFR2 and P0 for FGFR2b; Xu et al., 1998; De Moerlooze et al., 2000). We therefore used a conditional allele of FGFR2 (Yu et al., 2003) so that we could disrupt the gene postnatally but before mossy fibers form synapses on granule cells. For inactivation, we mated the conditional mutants to mice bearing a ubiquitously expressed transgene in which Cre recombinase was fused to the ligand binding domain of an estrogen receptor (Guo et al., 2002; Buffelli et al., 2003). The receptor had been mutated to prevent binding of the endogenous steroid while maintaining sensitivity to the synthetic ligand, tamoxifen. Thus, injection of tamoxifen to newborn pups or their nursing mother led to *FGFR2* deletion during the first few postnatal days.

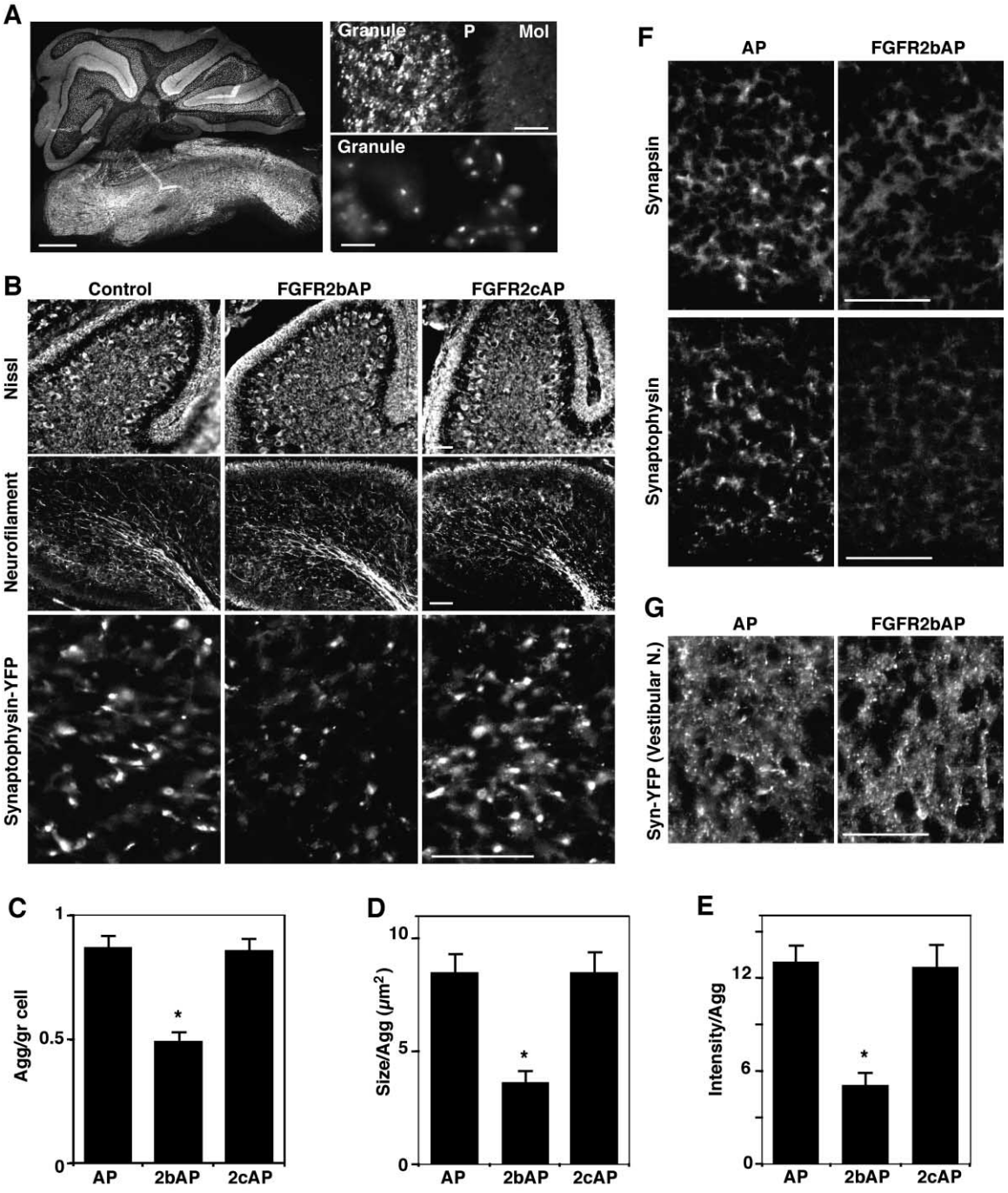

Figure 6. FGF-Dependent Presynaptic Differentiation of Mossy Fibers In Vivo

(A) (Left) Sagittal section of hindbrain from a P21 synaptophysin-YFP transgenic mouse, showing expression of YFP in hindbrain nuclei and in the granule layer of the cerebellum. (Right) Higher magnification views of cerebellum show punctate fluorescence in mossy fibers of granule layer. P, Purkinje cell layer; Mol, molecular layer. Bars are 500 μm, 20 μm, and 4 μm for the three panels.

(B) Cerebella of synaptophysin-YFP mice that were injected with 2 μg FGFR2bAP or FGFR2cAP on P3 and fixed on P8. Neither protein detectably affected the morphology of the cerebellum (assessed by Nissl staining) or the entry of mossy fibers (assessed with anti-neurofilament) as compared to untreated control. FGFR2bAP but not FGFR2cAP inhibited formation of aggregates of YFP-positive mossy fiber varicosities in the granule layer.

(C–E) Effect of FGFR2bAP and FGFR2cAP on the number of YFP-positive aggregates per granule cell (C), average aggregate size (D), and the mean intensity of YFP within aggregates (E). For quantitation, FGFR2AP-injected mice were compared to mice injected with AP alone. Bars show mean ± SEM for 50 sections from five mice per condition. *: differs from control at $p < 0.01$ by Tukey test.

(F) Effect of FGFR2bAP, injected as in (B), on synaptic vesicle aggregation in the granule cell layer of wild-type mice, assessed by immunostaining with anti-synapsin or anti-synaptophysin.

(G) Effect of FGFR2bAP on synaptic vesicle aggregation in the vestibular nucleus of synaptophysin-YFP mice. Injection and analysis as in (B). Bars in (B), (F), and (G) are 50 μm.

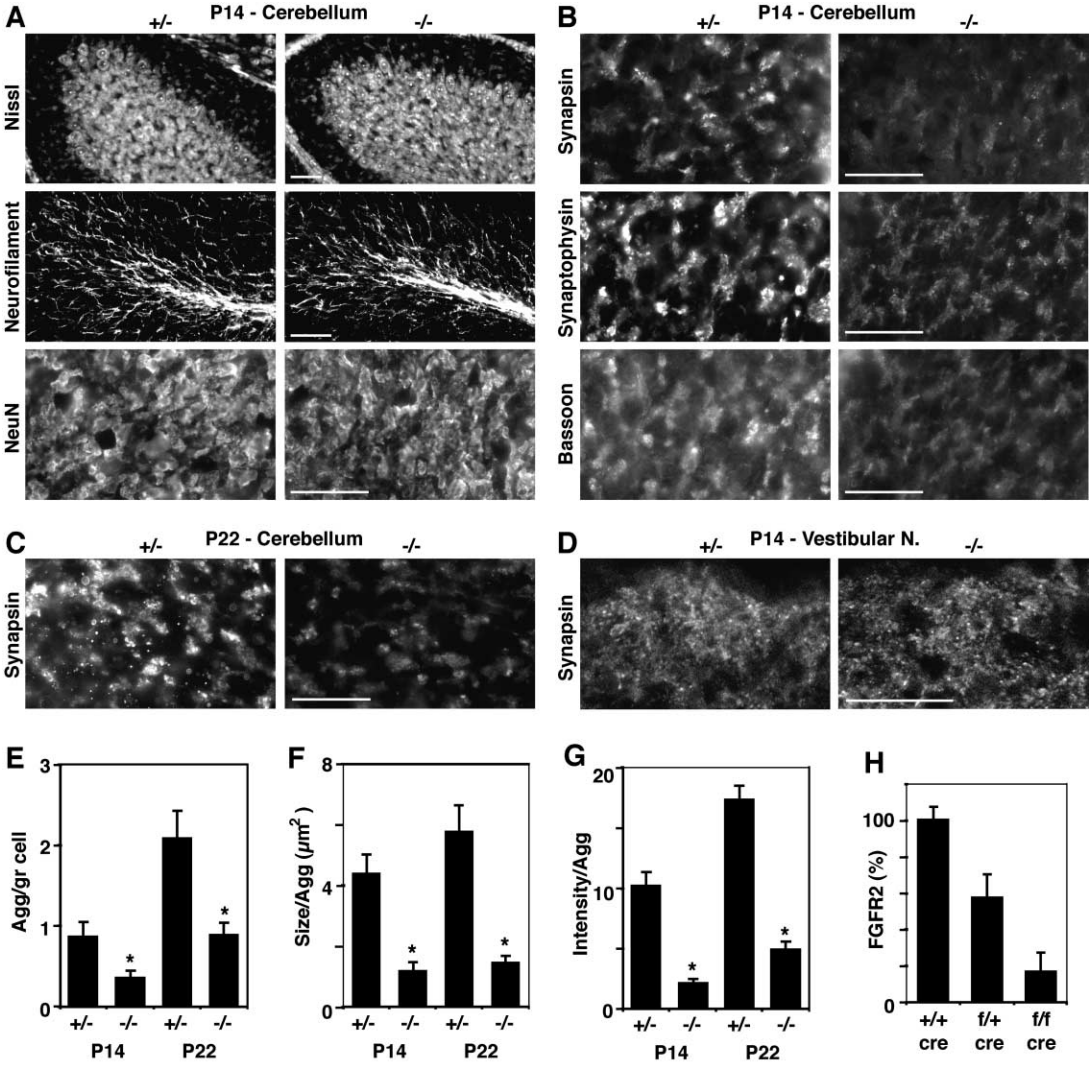

Figure 7. FGFR2-Dependent Presynaptic Differentiation of Mossy Fibers In Vivo

FGFR2 was inactivated postnatally by administration of tamoxifen to FGFR2$^{flox/flox}$; actin-cre-er mice.

(A) Inactivation of FGFR2 had no detectable effects on the morphology of the cerebellum (assessed by Nissl staining), the entry of mossy fibers (assessed with anti-neurofilament), or the density of granule cells (assessed with anti-NeuN).

(B and C) Inactivation of FGFR2 inhibited presynaptic differentiation in the granule cell layer of the cerebellum as assessed by staining for synapsin, synaptophysin, or bassoon. Animals were analyzed at P14 (B) or at P22 (C).

(D) Inactivation of FGFR2 had no detectable effect on vesicle aggregation in the vestibular nucleus. Bars are 50 μm for (A)–(D).

(E–G) Effect of FGFR2 inactivation on the number of synapsin-positive aggregates per granule cell (E), average aggregate size (F), and the mean intensity of synapsin immunofluorescence within aggregates (G). Lobule X was used for quantitation. Bars show mean ± SEM for 50 sections from five mice per condition. *: differs from control at $p < 0.01$ by Tukey test.

(H) FGFR2 in brain extract prepared from FGFR2$^{flox/flox}$; actin-cre-er, FGFR2$^{flox/+}$; actin-cre-er and FGFR2$^{+/+}$; actin-cre-er mice. All mice received tamoxifen postnatally from maternal milk; hindbrain and cerebellum were analyzed by immunoblotting at P14; films were scanned and density of bands calculated. Bars show values from three mice.

PCR of genomic DNA confirmed excision, and levels of FGFR2 protein were reduced by ~80% (Figure 7H and data not shown). Animals appeared healthy for at least three weeks.

Postnatal inactivation of FGFR2, like postnatal neutralization of FGF22-like factors, had no detectable effects on granule cell migration, cerebellar lamination, neurite ingrowth, or granule cell density (Figure 7A). However, presynaptic differentiation in the granule layer was substantially inhibited, as shown by staining for the vesicle protein synapsin and synaptophysin and the active zone protein, Bassoon (Figure 7B). Effects of FGFR2 inactivation, assessed by synapsin staining, were similar to those of FGFR2bAP and included decreases in the number, size, and intensity of synapsin-rich aggregates in the granule layer (Figures 7E–7G). Effects measured at P22 were similar to those observed at P14 (Figures 7C and 7E–7G). Thus, although we do not know whether FGFR2 inactivation inhibits or merely delays presynaptic differentiation, our results support the former possibility.

Finally, because tamoxifen was administered systemi-

cally in these experiments, FGFR2 was excised throughout the body. We were therefore able to use these animals to ask whether presynaptic differentiation was globally dependent on FGFR2-mediated signaling. FGFR2 excision had no detectable effect on vesicle aggregation in vestibular nuclei (Figure 7D), supporting the idea that FGF/FGFR2 signaling mediates presynaptic differentiation of specific neuronal subpopulations.

Discussion

FGF22 as a Presynaptic Organizer

Treatment of cultured neurons with an extract of mouse forebrain caused a redistribution of synaptic vesicles in their neurites, leading to the formation of clusters resembling those found in presynaptic varicosities. Using this redistribution as an assay, we purified a major active protein from the extracts and identified it as FGF22. We then obtained several lines of evidence in support of the idea that FGF22 is a target-derived organizer of presynaptic differentiation: (1) Recombinant FGF22 induced aggregation of synaptic vesicles in neurites, as assayed with stains for three vesicle components, synapsin, SV2, and synaptophysin (Figure 2A). (2) Vesicles in FGF22-induced clusters were capable of depolarization-dependent recycling, as monitored by uptake and release of FM1-43 (Figures 2B and 2F). (3) FGF22 also induced branching of neurites in a pattern that might contribute to formation of a terminal arbor (Figures 2A and 2E). (4) FGF22 exerted effects on both vesicle clustering and neurite branching at a physiological concentration (0.25 nM; Figure 2H). (5) Effects of FGF22 were direct (they were seen in low-density cultures highly enriched for a single neuronal type and lacking nonneuronal cells) and specific (they were unaccompanied by detectable effects on neuronal migration or survival or neurite length). (6) FGF22 is expressed by specific neuronal populations during the period that they receive synapses (Figure 4A). (7) One population that expresses FGF22, the cerebellar granule cells, receive their principal innervation from pontine and vestibular neurons, which express the main FGF22 receptor, FGFR2 (Figure 4B). (8) FGFR2bAP, which binds FGF22, inhibited presynaptic differentiation of pontine and vestibular axons, called mossy fibers, both in vitro (Figure 5) and in vivo (Figure 6). (9) Conditional deletion of FGFR2 impaired presynaptic differentiation of mossy fibers in vivo (Figure 7). Together, these results provide strong evidence that FGF22, or a closely related molecule, is a target-derived presynaptic organizer for cerebellar mossy fibers.

The FGF7/10/22 Subfamily

Although the evidence that FGF22 can promote presynaptic differentiation is strong, there is a caveat to the evidence that it does so in vivo. FGF7 and -10, the two closest relatives of FGF22 (Figure 3C), share its ability to promote vesicle clustering and neurite branching in vitro and are expressed by neuronal subsets in vivo. Moreover, the two methods that we used to demonstrate a role for FGF signaling—neutralization with FGFR2bAP and conditional deletion of FGFR2—would not distinguish among these three closely related FGFs. We suspect that FGF22 is the predominant organizing molecule in cerebellum because its RNA is expressed by granule cells at much higher levels than FGF7 or -10. FGF7 and -10 may, however, play predominant roles at other synapses. Testing these roles will require inactivating the FGFs rather than their receptors. Targeted mutants of the FGF7 and -10 genes have been generated (Guo et al., 1996; Min et al., 1998), but to date no studies of their synapses have been reported. Mutation of the FGF22 locus is underway.

The FGF Family

FGF family members play numerous roles in neural development, including neural induction, patterning of the neural plate, regulation of neuroblast proliferation and survival, and differentiation of neuronal subtypes (reviewed in Dono [2003]; Reuss and von Bohlen und Halbach [2003]). No neural roles have been reported, however, for members of the FGF7/10/22 subfamily. It is therefore tempting to propose a unique presynaptic organizing role for this subfamily.

On the other hand, at least some FGFs from other subfamilies were also active in our assay. Their activities differed, however, from those of FGF7, -10, and -22. FGF4, -6, and -9 induced vesicle clustering but not neurite branching, while FGF17 and -18 induced neurite branching but not vesicle clustering. FGF4, -6, and -9 all signal predominantly through the "IIIc" splice variants of FGFR1 and 2 (Ornitz et al., 1996), suggesting that the IIIc forms may also be involved in synaptic development.

Previous studies have shown that each FGF is expressed in a distinct pattern, and we have found that some of their activities are cell type specific. Because responses to FGFs are mediated by a large set of FGFR isoforms (at least seven alternatively spliced isoforms of four FGFRs, each with a distinct specificity) and modulated by heparan sulfate proteoglycans, variations in receptor and proteoglycan repertoire are likely bases for such cell type-specific responses. Thus, a picture emerges in which different FGF family members have distinct effects, are expressed by distinct cell types, and act on distinct subsets of targets. Thus, FGFs could be involved not only in synaptic differentiation per se but also in the specificity with which particular inputs synapse on particular targets.

Multiple Regulators of Presynaptic Differentiation

Several proteins have been proposed to be target-derived organizers of presynaptic differentiation in mammals (reviewed in Scheiffele [2003]; Yamagata et al. [2003]). One, laminin $\beta 2$, is largely confined to the neuromuscular synapse, but others are broadly distributed in brain. What is the relationship between these proteins and FGFs? One obvious possibility is that different organizers organize different synapses. However, two candidates, neuroligin and WNT7a, were previously implicated in formation of the mossy fiber synapse (Scheiffele et al., 2000; Hall et al., 2000), which we now show to be dependent on FGF22. We therefore believe that multiple organizers are involved in guiding formation and maturation of a single synapse.

Much of our thinking about synaptic organizing molecules is derived from the neuromuscular junction, where

nerve-derived z-agrin plays a predominant role in organizing numerous aspects of postsynaptic differentiation; there is currently little evidence for other nerve-derived postsynaptic organizers at this synapse (reviewed in Sanes and Lichtman [2001]). In contrast, presynaptic differentiation may be controlled in a more complex way. At the neuromuscular junction, motor nerve terminals form normally in embryos lacking laminin β2, but their maturation is severely compromised (Noakes et al., 1995; Sanes and Lichtman, 1999; H. Nishimune and J.R.S., submitted). Conversely, maturation of mossy fiber-granule cell synapses is delayed in mutants lacking WNT-7a, but their final differentiation is unperturbed (Hall et al., 2000). Thus, distinct target-derived factors may act together to organize presynaptic differentiation, with individual factors specifying appropriate sites for synaptogenesis, localizing subsets of presynaptic components, promoting functional maturation, and maintaining the release apparatus once it has matured. FGF22 and its relatives may be involved in any of these processes. An important next step in the effort to understand synaptogenesis will be to determine how multiple factors cooperate to organize presynaptic differentiation.

Experimental Procedures

Purification and Identification of Synaptic Organizing Molecules

Forebrains from 100 P7 mice were homogenized in 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris (pH 7.5), containing 0.1 mg/ml PMSF, then spun at 20,000 g for 30 min. The supernatant was dialyzed against buffer A (10 mM Tris pH 8.0), applied to a DEAE-Sepharose FF column, and eluted with a 0–500 mM linear NaCl gradient (AKTAprime FPLC System; Pharmacia). Active fractions were pooled, diluted in buffer A, applied to a 20 ml HiTrap Q column, and eluted with a 100–200 mM NaCl gradient. Active fractions were concentrated (Centriplus10, Amicon) and loaded onto a Superdex-200 HR10/30 gel filtration column, which was run with Buffer A plus 150 mM NaCl. Active fractions were dialyzed against 20 mM sodium acetate buffer (pH 5.0), applied to a MONO-S HR 5/5 column, and eluted with a 0–500 mM NaCl gradient. Active fractions were concentrated with Centricon 10 (Amicon), separated on a 4%–15% gradient SDS-polyacrylamide gel, and stained with Colloidal Blue Stain (Novex). The 20 kDa band was excised, digested with trypsin, and subjected to matrix-assisted laser desorption ionization mass spectrometry at the Keck Biotechnology Resource Laboratory, Yale University. Databases were searched with the detected masses using ProFound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

Motoneuron Assay

Motoneurons from E5 chick spinal cord were purified and cultured essentially as described by Henderson et al. (1996), using Metrizamide density gradient centrifugation. Motoneurons were chosen because they differentiate quickly in low-density culture; chicks were used instead of mice for convenience. Motoneurons were plated at 2000 cells/well in 8-well Lab-Tek Permanox chamberslides coated with poly-D,L-ornithine and laminin (Invitrogen). Cultures were maintained in L-15 medium with insulin, putrescine, conalbumin, progesterone, sodium selenite, and 2% horse serum. Proteins or extracts to be tested were added at the time of plating except where indicated.

For styryl FM dye imaging, cultures were loaded for 90 s with 10 μM FM1-43 in a modified Tyrode solution containing 107 mM NaCl, 47 mM KCl, 2 mM MgCl2, 10 mM glucose, 10 mM HEPES-NaOH pH 7.4, and 2 mM CaCl2. After washing for 10 min, cultures were treated with 4 mM or 90 mM KCl Tyrode (KCl plus NaCl held constant). Images were captured with a CCD camera (Photometrics) and analyzed with Metamorph imaging software (Universal Imaging).

Histology

Tissues were fixed in 4% paraformaldehyde infiltrated with 30% sucrose, frozen, and sectioned at 15 μm in a cryostat. Sections were blocked in 2% BSA and 0.1% TritonX-100 for 1 hr, followed by incubation with primary antibodies for 2 hr at room temperature or overnight at 4°C. Secondary antibodies were applied for 1 hr at room temperature and slides were mounted with p-phenylenediamine to retard fading. Images captured with a CCD camera (Optronics) were analyzed with Metamorph.

Sources of antibodies were as follows: anti-synapsin (a kind gift from Paul Greengard); anti-SV2 (Developmental Studies Hybridoma Bank); anti-Neurofilament (Sternberger); anti-MAP2 (Sigma); anti-NeuN (Chemicon); anti-Synaptophysin (Zymed); and anti-Bassoon (Stressgen). Neuro Trace 435 (Molecular Probes) was used as a fluorescent Nissl stain.

In situ hybridization was performed as described by Schaeren-Wiemers and Gerfin-Moser (1993) using digoxigenin-labeled riboprobes and alkaline phosphatase detection (Roche Molecular Biochemicals). The FGFR2 probe encoded the transmembrane region. The FGF10 probe was described by Bellusci et al. (1997). The FGF7 and FGF22 probes were generated by PCR from the 3′ untranslated regions.

Recombinant Proteins

A cDNA coding for mouse FGF22 was isolated from a mouse brain mRNA by RT-PCR. The coding region of the FGF22 cDNA excluding the sequence encoding the signal peptide was ligated to the glutathione-S-transferase (GST) gene in the pET-41 vector (Novagen). GST-FGF22 fusion protein was expressed in *E. coli* and bound to glutathione-agarose (Sigma). FGF22 was cleaved from GST by enterokinase, which was then removed on Ekapture-agarose (Novagen). Other FGFs were obtained from Peprotech or R&D Systems. AP and AP fusions were produced by transiently transfecting corresponding plasmids into COS cells (Ornitz et al., 1992). Secreted AP fusion proteins were purified by affinity chromatography on anti-AP agarose (Sigma).

Pontine/Vestibular Explants-Granule Cells Coculture

Pontine or vestibular nuclei dissected from P0.5 wild-type or synaptophysin-YFP (see below) transgenic mice were cut into pieces and cultured in 8-well Lab-Tek Permanox chamberslides coated with poly-D,L-ornithine and laminin (Baird et al., 1992). The explants were cultured for 48 hr in serum-free medium (Yuzaki and Mikoshiba, 1992) with or without granule cells. Dissociated granule cells were prepared as described by Yuzaki and Mikoshiba (1992) from P5–P9 mouse cerebellum and added to the explants at 50,000 cells per cm^2. Recombinant proteins were added to the culture media at the time of plating.

Animals

Transgenic mice in which a synaptophysin-YFP fusion protein was expressed under the control of regulatory elements from the *thy-1* gene (Feng et al., 2000; De Paola et al., 2003) were generated by standard methods. For use in this study, we selected a line (line 10) in which vestibular and pontine neurons were YFP positive by P0–P2. Actin-cre-er (Guo et al., 2002) transgenic mice and conditional FGFR2 mutants (Yu et al., 2003) were described previously. Tamoxifen was administered by intraperitoneal injection to newborn pups (0.1 mg on P0; Buffelli et al., 2003) or to their nursing mothers (1 mg/day for 5 days starting on the day of delivery; Leone et al., 2003). Genotypes and efficacy of tamoxifen treatment were confirmed by PCR (Yu et al., 2003). FGFR2 and FGFR2b antibodies were from Santa Cruz and R&D systems, respectively.

For treatment with blocking reagents, wild-type or synaptophysin-YFP pups were anesthetized by hypothermia on P3. Two micrograms fusion protein in 2 μl PBS was injected at 0.6 mm rostal and 0.8 mm lateral to bregma and 2 mm deep to the skull surface. The optimal location was determined in preliminary experiments by Trypan Blue injections. After surgery, pups were revived on a heating pad then returned to their mother. The pups were sacrificed at P8 for analysis.

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